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Use of an Ethanol-Driven Pressure Cell to Measure Hydrostatic Pressure Response of Protein-Stabilized Gold Nanoclusters

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14. ABSTRACT
In this investigation, an improved method to study the fluorescence enhancement of bovine serum albumin (BSA)-stabilized gold nanoclusters (BSA:Au_{25}NCs) at low pressures is introduced. This method uses an ethanol-driven pressure cell (PC) incorporated into a spectrofluorometer to measure the pressure sensitivity of BSA:Au_{25}NCs. The ethanol-driven PC allows for the application of hydrostatic pressure at a much lower range and with higher repeatability than current methods of pressure testing, which involve using a Diamond Anvil Cell (DAC). Results demonstrate that the pressure response of BSA:Au_{25}NCs studied in the fluorometer-based PC exhibited a linear fluorescence response upon pressure loading. These data correlated well with the previously published results obtained using the DAC technique at pressures above 200 MPa. Overall, the PC used in this study was better suited to detect pressure sensitivity at pressures under 200 MPa, while exhibiting much improved repeatability over current DAC techniques, making it ideal for testing the pressure sensitivity of metal nanoclusters.

15. SUBJECT TERMS
protein-stabilized nanoclusters, pressure response, pressure cell, ethanol-driven, bovine serum albumin

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1. Introduction and Background

Noble-metal nanoclusters (NCs) are typically less than 2 nm in diameter and have been extensively researched as a new type of fluorophore.[1–3] Unlike nanoparticles (NPs), whose size is comparable with the electron mean-free path (e.g., 20 nm for gold [Au]) and whose absorbance is based on surface plasmon resonance,[4] noble-metal NCs have sizes comparable with the Fermi wavelength (~0.5 nm for Au and silver [Ag]) and exhibit fluorescence based on discrete electronic states.[5] Because of their small size and molecular-like properties, noble-metal NCs are typically regarded as the "missing link" between atomic and NP behavior.[6] NCs are synthesized via 2 distinct routes, chemically derived and bio-enabled. The chemically derived approaches are achieved either through the etching of larger NPs or by de novo synthesis using metal salts followed by the addition of stabilizing ligands.[7] In bio-enabled synthesis, biological molecules such as, protein (P-NCs) or DNA, both reduce and stabilize NCs during syntheses (Fig. 1) resulting in highly biocompatible fluorescent markers, suitable for immediate use in vivo.[5,7] This is a property unique to biosynthesized NCs as semiconductor quantum dots and chemically derived NCs require further capping or shielding, with limited success due to metabolism and accumulation, before use in vivo.[4,5] Furthermore, several proteins have been shown to retain native function post-NC synthesis and the NCs have been used to observe this activity.[8–11] Thus, P-NCs are able to report on the stabilizing protein's activity without interfering with the process making them a "silent sensor".

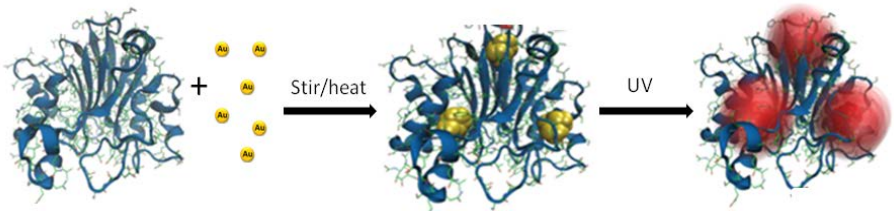

Fig. 1 Schematic of standard biomediated P-NC synthesis process

Recently, Zhang et al. were able to demonstrate that P-NCs were able to report mechanical stresses in addition to functional activity using BSA-stabilized gold nanoclusters (BSA:AuNCs).[12] The BSA:AuNCs were found to exhibit a pressure dependent fluorescence increase. These data demonstrated that the increase in fluorescence was linear, theoretically enabling the back calculation of observed pressure in a system. The pressure dependence was hypothesized to be due to compression of the protein's ligands around the NC.

The results obtained by Zhang et al. were found using a symmetric diamond anvil cell (DAC) (Fig. 2).[12,13] In this system, the sample is placed between parallel faces of opposed diamond anvils. Pressure is applied to the sample by pushing the diamonds together using a screw mechanism, where calibration of the applied pressure is determined using a small ruby crystal or a similarly well-characterized material placed within the sample chamber as an internal standard.[13] This method is typically used for application of extremely high pressures in the gigapascal range as there is difficulty in locating standards that are responsive at lower pressures. Thus, the lack of sensitivity at low pressures and requirement of an external indicator make the DAC an inefficient and uneconomical choice for testing at sub-gigapascal pressures.

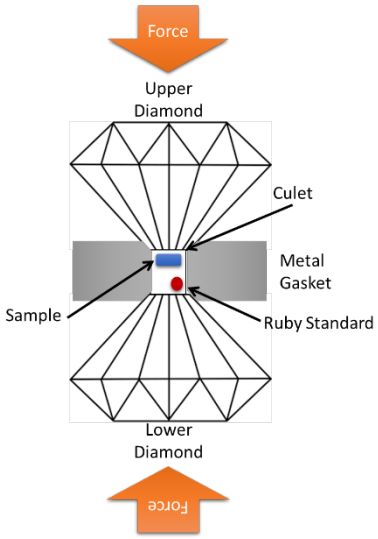

Fig. 2 Diagram of high-pressure DAC instrumentation and methodology

The purpose of this study was to examine the BSA:AuNC pressure response utilizing the pressure cell (PC) and to compare the effectiveness of the PC methodology to the current state of the art DAC technology. The PC system is intriguing due to its various desirable properties. First, no internal standard is needed to accurately quantify pressure, and as this system is ethanol driven, specific pressures can be more systematically and reproducibly measured. Moreover, the PC fits inside the sample chamber of a fluorescence spectrometer, thus allowing the quantification of pressure response in real time via either a wavelength scan or single point measurement.[14] Furthermore, the ethanol-driven cell allows for pressure application in the megapascal or even kilopascal range, which is in contrast to the gigapascal range that limits DAC technology. We hypothesize that the PC will enable facile and repeatable investigation of the pressure response of protein-stabilized nanoclusters at lower pressure ranges.

To validate the operation of this PC system, BSA:AuNCs were synthesized and the pressure response due to increasing pressures was investigated in the range of 0–400 MPa. This response was then compared to the results obtained by Zhang et al. in the same pressure range.

2. Synthesis of Materials

2.1 Synthesis of BSA-Stabilized Gold Nanoclusters

A solution of BSA:AuNCs was synthesized using published methods.[9] Briefly, aqueous chloroauric acid ($HAuCl_4$) (5 mL, 10 mM, 37 °C) was added to BSA (5 mL, 50 mg/mL, 37 °C) and stirred vigorously for 2 min. Sodium hydroxide (NaOH) (0.5 mL, 1 M) was subsequently added to raise the pH to approximately 12, and the reaction was left to incubate at 37 °C for 12 h until the solution turned a dark brown color. The resulting BSA:AuNCs were centrifuged to remove excess gold salt and lyophilized for storage. The resulting NCs exhibit bright red fluorescence and are composed of 25 Au atoms stabilized by BSA protein and are approximately 2 nm in diameter as confirmed by transmission electron microscopy (TEM) (Fig. 3).

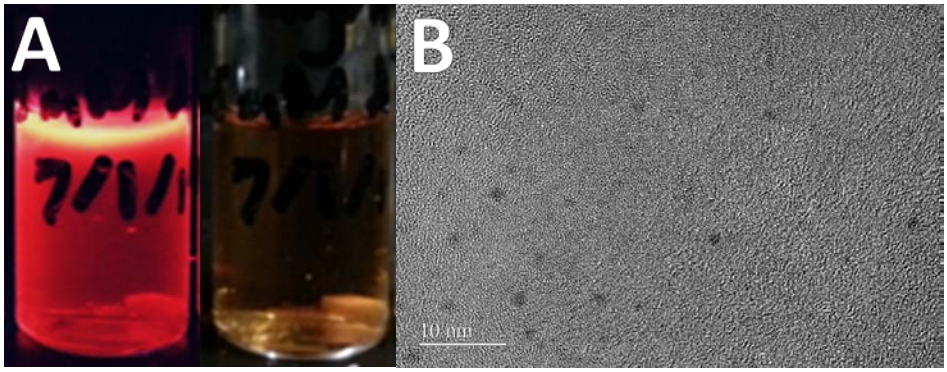

Fig. 3 BSA:AuNC characterization. A) BSA:AuNCs illuminated by ultraviolet (left) and bright field (right) illumination. B) TEM of BSA:AuNCs.

2.2 Pressure Cell Operation

To apply pressure to the aqueous BSA:AuNCs, an ethanol-driven PC manufactured by ISS was used (ISS, Inc., Champaign, IL). This PC was designed to fit into a Horiba Jobin Yvon Fluorolog spectrofluorometer and can be used to apply hydrostatic pressure in the range of 0–400 MPa to an aqueous sample. The PC used for hydrostatic pressure testing is shown in Fig. 4.

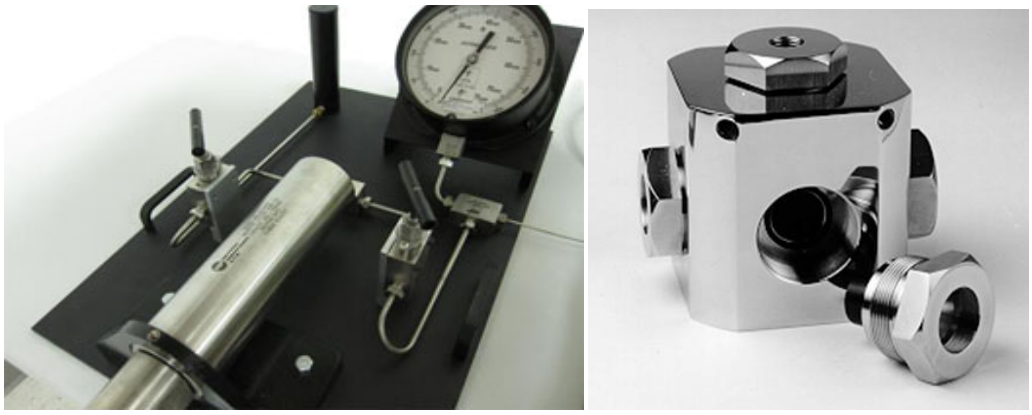

Fig. 4 Ethanol-driven hydrostatic PC

For pressure testing, lyophilized BSA:AuNCs were resuspended in ultrapure deionized water (Millipore Milli Q) at a concentration of 10 mg/mL. The BSA:AuNCs solution was placed in a circular quartz cuvette and fitted with a sealed plastic cap to allow for transfer of pressure to the sample. Special care must be taken to ensure that no air bubbles are present in the sample cuvette and that the cap is able to move up and down the neck of the cuvette to ensure accurate transfer of pressure to the sample. The sample cuvette was then placed into the supplied cradle and inserted into the PC. The PC was then filled with spectroscopic-grade ethanol until the sample cuvette and cap were fully immersed. The top nut of the PC was then screwed into the PC and tightened to 50 ft-lb using a click-type torque wrench. It is important to ensure that a small amount of ethanol from the PC leaks into the tightened top nut to ensure that no air is present in the cell. A connecting nut is then screwed into the top nut and tightened; again it is important to ensure that ethanol leaks into the connecting nut to ensure the absence of air pockets.

A schematic of the ethanol-driven PC used in this investigation is shown in Fig. 5. The ethanol used to drive the pressure is initially stored in a reservoir (1) and was pumped into the pressure generator (3) using a piston mechanism by turning a large wheel. The reservoir was filled by opening the reservoir valve (2) while ensuring that the PC valve (4) was closed. The wheel was spun counterclockwise until the piston was fully retracted, signaling that the pressure generator was completely filled. The reservoir valve was then closed and the PC valve was opened. The wheel was slowly turned clockwise to drive the ethanol from the pressure generator to the PC hose (7) until a steady drip of ethanol was observed from the end of the hose, indicating that the hose was fully purged of air. The PC hose was then attached to the connecting nut on top of the PC and tightened.

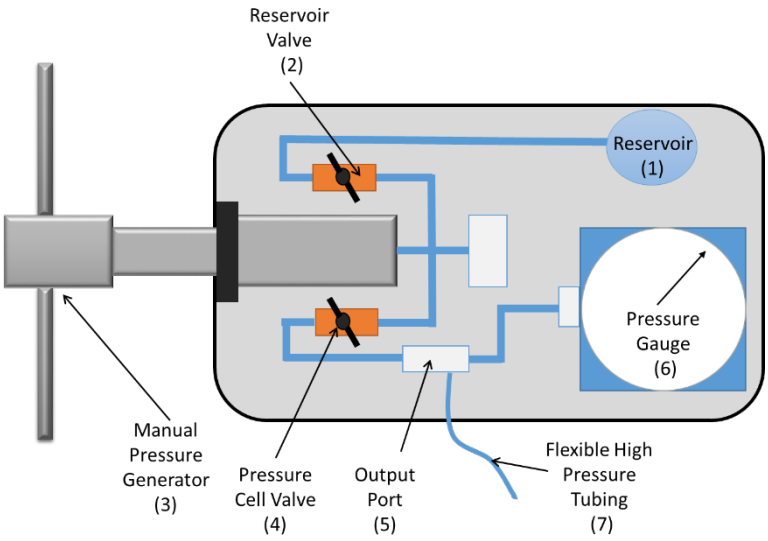

Fig. 5 Schematic of ethanol-driven PC

Once the PC was primed, the cell was placed into the Horiba Fluorolog Spectrofluorometer to measure the emission spectra. An excitation wavelength of λex = 470 nm was used. The emission spectrum was measured in the range of 540–750 nm in order to fully capture the emission peak at approximately 635 nm. An excitation and emission slit width of 5 nm was used in all trials. To increase signal to noise ratio, 5 scans of the emission spectrum were taken and averaged.

To measure the pressure induced change in the fluorescence intensity of the BSA:AuNCs, a measurement of the fluorescence emission spectrum was taken at pressures of 0–400 MPa in 50 MPa intervals. Pressure was increased by slowly turning the wheel attached to the driver reservoir clockwise. A time period of at least 5 min was allowed between increasing the pressure and measuring the emission spectrum to ensure that the system reached equilibrium. Once a pressure of 400 MPa was reached, the pressure was relieved by slowly turning the wheel counter-clockwise, again making sure to allow a time period of 5 min at 50 MPa intervals to prevent damage to the cell.

Once the pressure was completely relieved and the driving reservoir was filled, the hose was disconnected from the PC. The valve connecting the driving reservoir to the PC hose was then closed, and the valve connecting the driving reservoir to the ethanol holding tank was opened. The ethanol in the driving reservoir was then completely emptied into the holding tank by turning the wheel clockwise until the piston was fully inserted. The connecting nut and top nut were then removed from the PC, and the sample cuvette was removed, emptied, and rinsed.

3. Results and Discussion

A baseline measurement of the excitation and emission spectrum of the BSA:AuNCs was first taken without any applied pressure (Fig. 6). The emission spectra shows a peak at approximately 625 nm with dual excitation peaks at around 390 and 480 nm. These spectra are consistent with the fluorescence spectra of BSA:AuNCs found in literature.

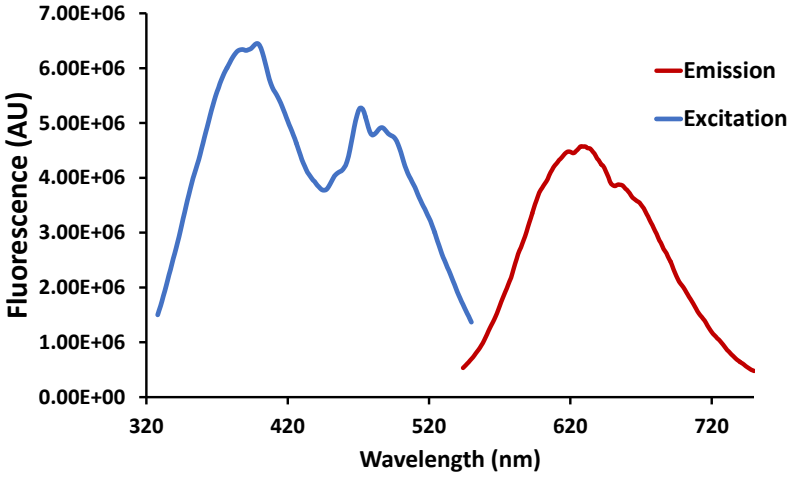

Fig. 6 Excitation (blue) and emission (red) spectra of BSA:AuNCs

Similar to the results observed by Zhang et al., the BSA:AuNCs displayed an increase in fluorescence intensity that was directly proportional to the increase in pressure.[12] We were able to observe a linear relationship over the full range of pressure tested (Fig. 7). The rate of fluorescence increase for the BSA:AuNCs is approximately 0.06%/MPa with a linear correlation of 95%, resulting in an increase in fluorescence intensity of approximately 23% at 400 MPa.

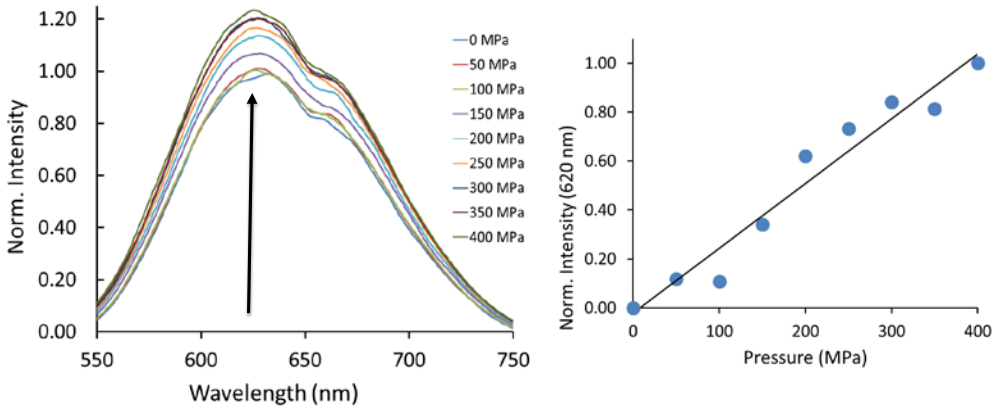

Fig. 7 Fluorescence response of BSA:AuNCs to hydrostatic pressure changes in the range of 0–400 MPa

In comparison to the results obtained with DAC technology, a similar trend can be seen wherein a pressure induced increase in fluorescence intensity of the BSA:AuNCs exists, resulting in an increase of just over 20% at 400 MPa.[12] These results indicate that there is a good correlation between the published results and results obtained using the ethanol driven PC. Moreover, the DAC study is a negligible increase in pressure induced fluorescence enhancement at pressures below 200 MPa; however, the results obtained using the PC indicate a steady rise of fluorescence intensity even at these low pressures.

4. Summary and Conclusions

In this investigation, an ethanol-driven PC integrated into a fluorescence spectrometer was employed to determine the pressure sensitive fluorescence enhancement of BSA-stabilized 25-atom gold nanoclusters at pressure ranges in the megapascal regime. BSA:AuNCs were successfully synthesized and subjected to static pressures ranging from 0–400 MPa in 50 MPa intervals, while the emission spectrum was measured using the spectrofluorometer.

The pressure response of the BSA:AuNCs obtained using the ethanol-driven PC correlated well with the published results in the range of 200–400 MPa. However, unlike the published results that show negligible pressure response at pressures below 200 MPa, results obtained using the PC showed a steady pressure response at these lower pressures that correlated with the overall linear response. These results support the validity of using a PC to investigate the pressure-sensitive fluorescence enhancement of protein-stabilized metal NCs. Further, they seem to indicate that the ethanol-driven PC method is better suited to measuring pressure responses at low pressures (<200 MPa).

Future work will focus on using this system to investigate the pressure response of a multitude of P-NCs consisting of various proteins, metals, and sizes to determine the mechanism of pressure sensitivity and utilize them for pressure-related applications.

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List of Symbols, Abbreviations, and Acronyms

Ag	silver
ARL	US Army Research Laboratory
Au	gold
BSA	bovine serum albumin
DAC	diamond anvil cell
NC	nanocluster
NP	nanoparticles
P-NC	protein nanocluster
PC	pressure cell
TEM	transmission electron microscopy

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